

Screening of Groundnut Germplasm for Foliar Fungal Diseases and Population Structure Analysis Using Gene Based SSR Markers

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Authors' contributions

This work was carried out in collaboration among all authors. Authors PB and ST designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author RST managed the analyses of the study. Authors MKT and RSS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Groundnut (*Arachis hypogaea* L.) is an excellent cash crop having multipurpose uses of each plant part i.e., direct consumption, cooking oil and a rich source of protein feed for animals. Rust and late leaf spot (LLS) are two major foliar fungal diseases of groundnut and can cause yield loss up to 70%. Use of fungicide is costly approach and it is not environment-friendly also, therefore breeding new cultivars with genetic resistance is sustainable, environment-friendly and cost effective approach. Screening and identification of resistant cultivar is one of the primary objective for groundnut improvement. Use of molecular markers, particularly gene based, are user friendly and cost effective approach to identify groundnut resistant genotypes. The present investigation aimed to screen groundnut germplasm using allele specific primers for foliar fungal diseases (LLS and rust). The study consisted 30 uncharacterized germplasm lines and 4 check

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varieties of Groundnut. A set of 4 gene based SSR markers were selected for screening of groundnut germplasm for foliar fungal diseases i.e., LLS and rust. A total of 14 alleles were identified with an average of 3.5 alleles per locus for polymorphic SSR markers. The gene diversity and Polymorphic Information Content (PIC) values varied between 0.3972-0.5778 with an average of 0.47 respectively. The genetic relationships among Groundnut genotypes are presented in SSR based UPGMA tree. Principle Co-ordinate analysis (PCA) based on origin formed 4 major population groups. The population structure of the 34 Groundnut genotypes was estimated using STRUCTURE v2.3.3 software based on SSR markers. The optimum K value was determined by using Structure Harvester, where the highest peak was observed at delta K = 2. The number of sub populations (K) was identified based on maximum likelihood and delta K (dK) values, with two core and pure groups and an admixture group. Current study identified four germplasm i.e., AH8054, CS21181, CS708, Akola White to be used as foliar disease resistant cultivar for groundnut hybridization and improvement.

Keywords: Groundnut germplasm; SSR markers; population structure; PCoA.

1. INTRODUCTION

Groundnut or peanut (*Arachis hypogaea* L.) is one of the most imperative oilseed crop cultivated in the semi-arid tropics. It is an important self-pollinated oilseed crop grown in more than 100 countries with different agro-climatic conditions on about 26.5 million ha with total production of 43.9 million tons and productivity of 1654 kg ha⁻¹ in 2014 [1]. Groundnut is one of the major crops grown in Kharif season and Gujarat is the prominent state growing groundnut followed by Andhra Pradesh, Tamil Nadu, Rajasthan and Karnataka. The cultivated groundnut (*Arachis hypogaea* L.) is originated in South America and it is an allotetraploid ($2n = 4x = 40$) with a genome size of 2891 Mbp, originated through a single hybridization and polyploidization event. Peanut sub-genomes contain about 64% repetitive sequence [2], therefore less chances of polymorphism has been reported. In India, it is primarily an important oilseed crop but growing realization of confectionary qualities traits in kernel, it is now being realized as a food and fodder crop. It is valued as a rich source of energy in form of oil (48-50%) and protein (25-28%) in the kernels. It provides 564 kcal of energy from 100 g of kernels [3]. Besides this, it contains many health beneficial nutrients such as minerals, vitamins and antioxidants. Groundnut haulms provide nutritious fodder for livestock. It contains protein (8-15%), lipids (13%), minerals (9-17%) and carbohydrate (38-45%) higher than cereal fodder. The digestibility of nutrients in groundnut haulm is around 53% and that of crude protein 88% when fed to cattle.

Cultivated peanut is mainly grown in the semi-arid tropics region by low income farmers. As a result, crop productivity has been adversely

challenged by several biotic and abiotic stresses. There are several major abiotic and biotic stresses which cause significant yield loss in groundnut production. The major biotic stress factors include early leaf spot (*Cercospora* spp.), late leaf spot (*Phaeoisariopsis personata*), rust (*Puccinia arachidis*), mottle virus etc. In addition, aflatoxin contamination drastically affects product quality and greatly reduces value of the crop plant along with grains. Among biotic stresses, foliar fungal diseases including three major foliar diseases, namely: early leaf spot (*Cercospora arachidicola* Hori), late leaf spot and rust (*Puccinia arachidis* Speg.) are the most widely distributed and economically important diseases of groundnut. Foliar fungal diseases are the major production constraints of groundnut worldwide wherever the crop is grown. These diseases can cause more than 70% loss in yield besides adversely affecting the quality of the produce (pods, seeds and haulms) [4]. Late leaf spot is a major and widely distributed disease. It can cause defoliation and reduce pod and fodder yields about 50% and adversely affect quality of its produce [5]. Rust is also economic important disease causing yield losses in range of 10 - 52%, in addition to a decline in seed quality [5]. Foliar diseases can be control by chemical measures but they increase costs of production thus beyond the reach of small and marginal farmers and also pollute the environment. Chemical control measures are available but they increase production costs by 10% [6] and are beyond the reach of small and marginal farmers. Considering above facts in mind, development and growing of resistant cultivars is the best viable option to minimize economic losses of farmer and maintains good quality of the product. Conventional breeding has been the major avenue for providing modern groundnut

cultivars to farmers and has been successful in some crops but groundnut has lagged behind due to lack of knowledge about molecular markers linked to traits of interest. Significant progress in the area of molecular breeding increased the output of breeding approaches, especially where phenotypic selection is difficult, expensive and more importantly lack accuracy. Modern breeding methods *i.e.* molecular breeding may be helpful for solving these problems.

Marker-assisted selection is an important tool to enhance tolerance/resistance to these stresses and has the potential to enable faster and larger gains through genetic improvement of popular varieties [7]. The transfer of targeted traits has been completed in 2-3 years through marker-assisted backcrossing (MABC) as opposed to 6-8 years needed with conventional methods [8]. Over the last few years, about 5000 SSR markers have been developed for groundnut [9,10,11]. Zhao et al. [12] has identified a total of 135,529 and 199,957 SSRs from the 1,084.3 and 1,353.8 Mb genomic sequences of *A. duranensis* and *A. ipaensis*, respectively. Identification of resistant sources and knowledge of components and mechanism of resistance are the prerequisite for the success of disease resistance breeding programs. Insufficient disease incidence also complicates the selection of resistant plants in field experiments [13], Mondal, et al. [14]. Chaudhary et al. [15] used a set of 340 diverse peanut genotypes and screened for LLS and rust resistance and yield traits across three locations in India under natural and artificial disease epiphytotic conditions. The study revealed significant variation among the genotypes for LLS and rust resistance at different environments. Recently Chu et al. [16] developed an RIL population from crossing Florida-07 × GP-NC WS 16 and utilized this population to map QTLs associated with ELS and LLS resistance. Screening and identification of germplasm and advance breeding lines for foliar fungal diseases is one of the primary objective for resistant breeding. For late leaf spot and rust study of Pandey et al. [18] has provided allele-specific PCR-based markers using QTL-seq approach. These newly developed markers are cost-effective and very easy to genotype for developing improved groundnut lines with enhanced resistance to LLS and rust. The present investigations were conducted to screening groundnut germplasm line(s) using gene based SSR markers and identification of

superior germplasm for higher yield and foliar disease resistance.

2. MATERIALS AND METHODS

The plant materials consisted 30 uncharacterized germplasm lines and 4 varieties of Groundnut. Uncharacterized germplasm along-with check varieties were received from DGR, Junagadh (Table 1). Check varieties for the experiment includes GPBD4, foliar disease resistance and a high yielding variety; KDG128, foliar disease resistance and a high yielding variety; JGN3, variety released from Madhya Pradesh and sensitive to foliar diseases and Gangapuri, old local variety sensitive for foliar disease.

2.1 Genomic DNA Isolation and Genotyping with Gene Based Markers

About 3 to 5 young leaves from all the 34 groundnut genotype were sampled from the 20 days old seedling grown in the field. Leaf samples were collected from first three plants for uniformity and disinfected by ethanol wiping to remove infections of diseases. The genomic DNA was extracted using CTAB method [18] with minor modification. The quality of the DNA was checked on 1% agarose gel and the DNA concentration was estimated with the micro volume spectrophotometer (Helix Biosciences, New Delhi, India). The DNA concentration for use in polymerase chain reaction (PCR) was diluted to 20-30 ng/μl.

A set of 4 allele specific markers were selected for screening of groundnut germplasms for foliar fungal diseases *i.e.*, LLS and rust [17] (Table 2). The primers were synthesized by Eurofins Genomics India Pvt. Ltd. Polymerase chain reaction was performed in 10 μl reaction mixture comprising of 1X PCR buffer, 0.1 U Taq DNA polymerase, 1 μl dNTP (1 mM), 0.5 μl of forward and reverse primers each (10 pM) and 2 μl (20 ng/μl) of genomic DNA in a thermocycler (Bio-Rad, USA). The PCR protocol comprised of initial denaturation step of 94°C for 3 min followed by 35 cycles of 94°C for 1 min, annealing at 55°C for 30 sec, elongation at 72°C for 1 min with final extension at 72°C for 10 min. The PCR products were resolved on 3% agarose gel at 120V for 2-3 hrs and documented using UVP, Gel Documentation (Thermo Fisher Scientific).

Gel scoring was done by base pair analysis using ladder based on banding pattern. Data

Table 1. List of groundnut germplasm lines and their country of origin

Country of origin	Germplasm line
India	CS23, CS50, CS709, CS818-2, CS850-1, CS888, CS2118-1, CS2377, AS7808, AH7999, AH8054, AH7516, Akola White, 51-44, Erect Peanut, GPBD 4, KDG128, Gangapuri, JGN 3
USA	Southern Cross, USA53, Wo Rte Stu Kei, You Kalch,
SUDAN	A6, AH8312, Colorado Manfre
Taiwan	Tainan#1,
Argentina	TIPO, U2-24-6, U4-7-9, U4-7-17
Ghana	EC21010
UNK	10-1
UN	AH6644

Table 2. Allele specific primers used for screening of foliar fungal diseases of groundnut [17]

Trait	Marker name	Forward sequence	Reverse sequence	Annealing temp (°C)	Size (in bp)
Rust	GMRQ517	TGTACCTGAAAT GCAAGTTGAGAC	AATGTATGTGT GTTGGGCC	59	150
Rust	GMRQ786	AACATTGTAACA CTCACCTGGCTA	TCATGCTTGAA C TGTGCCTC	59	200
Rust	GMRQ843	AGCCTTGCGACT AGGTTTCAT	CATGGTGAGA GACGCGTAAG	59	200
LLS	GMRQ975	GGTATCATGATG AATTTTTAGAAGA CTAGG	GAAATTTGGC TT TGGGTTCA	59	150

sheet was prepared to run in population structure and allele pattern A/A was used if band was in the upper side and pattern B/B was used if the band was in the lower side, in heterozygous condition banding pattern was A/B and in case of no amplification -/- was used.

2.2 Molecular Screening and Population Structure Analysis

Based on the allele size the genetic profile of 34 groundnut genotypes was scored using SSR markers. The observations were recorded for major allele frequency, polymorphism information content (PIC) and genetic distance by using Unweighted Pair Group Method for Arithmetic average (UPGMA) tree using Power Marker v3.25 software [19] and the dendrogram was constructed using MEGA 6.0 software [20]. SSR data was again subjected to cluster analysis followed by bootstrap analysis with 1000 permutations for all the genotypes using Mega 6.0 software. The population structure for 34 Groundnut genotypes comprising both germplasms and released check varieties was inferred using Structure 2.3.4 software [21]. The structure outputs were visualized using Structure Harvester from which Evanno plots were constructed Earl, DA and Von Holdt, BM. [22]. Principle Co-ordinate Analysis (PCoA) based on origin was recorded using GenAlex software.

3. RESULTS AND DISCUSSION

3.1 Phylogenetic Analysis and PIC Information

A set of 4 allele specific primers were amplified in all the 30 groundnut germplasms along with 4 check varieties. A total of 14 alleles were identified with an average of 3.5 alleles per locus for polymorphic SSR markers. The gene diversity and Polymorphic Information Content (PIC) values varied between 0.3972-0.5778 (GMRQ975 to GMRQ517) with an average of 0.47 respectively (Table 3). The primer which showed highest gene diversity and PIC values was GMRQ517 while the lowest gene diversity and PIC values was observed for the primer GMRQ975. The major allele frequency varied between 0.53 (GMRQ517) to 0.67 (GMRQ786) with a mean value of 0.61.

The genetic relationships among Groundnut genotypes are presented in SSR based UPGMA tree (Fig. 1). All the genotypes are grouped into 7 clusters. The genotypes in Cluster 1: TIPO, AH7608, CS23, JGN3, 51-44; Cluster 2: EC21010, AH6644, U2246, CS60, AH6312, AH7999; Cluster 3: KDG28, CS8180, TINAN WOR, TE STU KB, Gangapuri; Cluster 4: YOU KAICH, SUTHERN CROSS; Cluster 5: GPBD4,

AH8054, CS21181, CS708, Akola White; cluster 6: Colorado Manfre; Cluster 7: AH7618, CS2377, A6, USA53, CS8501, ERECT PEANUT, 101 U4717 and CS888 NC1 were forming group other than check varieties. Cluster 1 is forming group with JGN3 variety that is very sensitive to foliar fungal diseases, whereas cluster 2 is forming separate group having no check variety. Cluster and 3 is grouped with KDG128 and Gangapuri hence showing moderately sensitive genotypes whereas clusters 4, 6 and 7 are forming separate groups having no check variety. Cluster 5 is grouping with GPBD 4 hence may be resistant to foliar fungal diseases. The clusters based on SSR markers have been found to have relationship with sensitivity to foliar disease resistance. Most of the genotypes with the similar

degree of foliar fungal disease resistance were clustered into same group.

3.2 Principle Co-ordinate Analysis for SSR

Principle Co-ordinate analysis (PCA) based on origin formed 4 major population groups. Group 1 included accessions from India, Argentina, USA and Taiwan, Group 2 included accessions mainly from India, Gambia, USA, Argentina and United Kingdom. Third group consisted of accessions from India, USA, United Kingdom, Argentina and the fourth group included accessions were mainly from India, USA. The accessions in all the 4 groups included India. It means all the varieties of India are having highly diversified characteristics (Fig. 2).

Table 3. Allele specific SSR markers presenting Major Allele Frequency (MAF), number of alleles, gene diversity and Polymorphic Information Content (PIC) in groundnut using power marker v3.25 software

Marker	MAF	Genotype number	Allele number	Gene diversity	PIC
GMRQ517	0.5294	4.0000	4.0000	0.6315	0.5778
GMRQ786	0.6765	3.0000	3.0000	0.4896	0.4400
GMRQ843	0.6176	4.0000	4.0000	0.5536	0.5022
GMRQ975	0.6176	3.0000	3.0000	0.4931	0.3972
Mean	0.6103	3.5000	3.5000	0.5420	0.4793

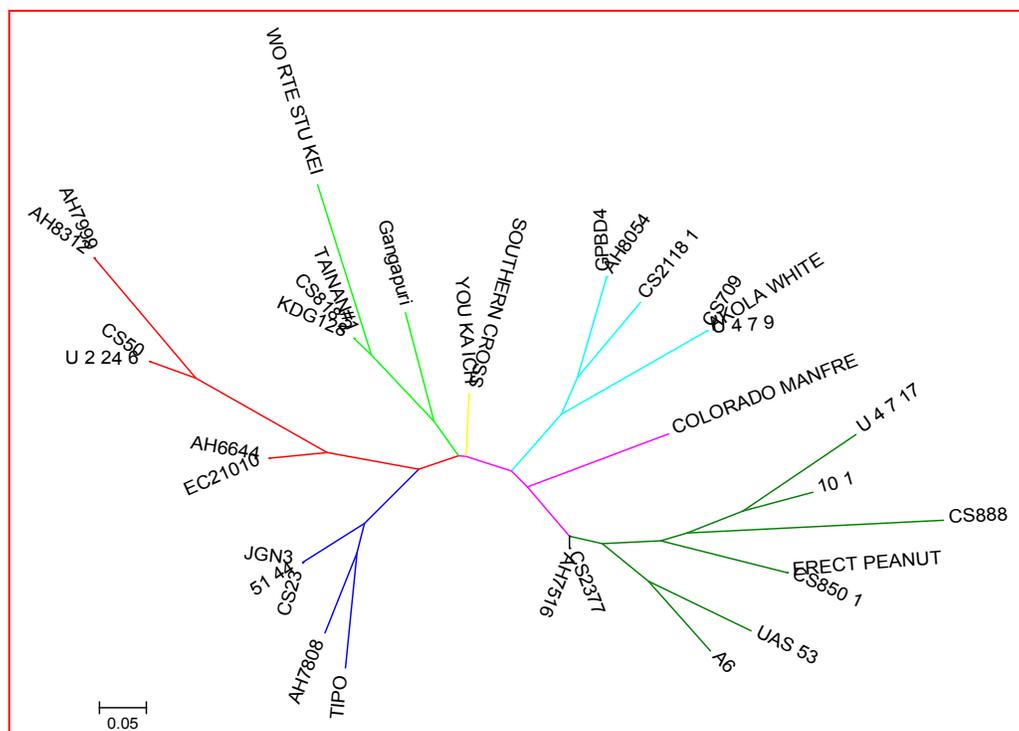


Fig. 1. Dendrogram of 34 groundnut germplasm lines based on banding pattern analysis of gene based SSR markers using MEGA 6.0 software

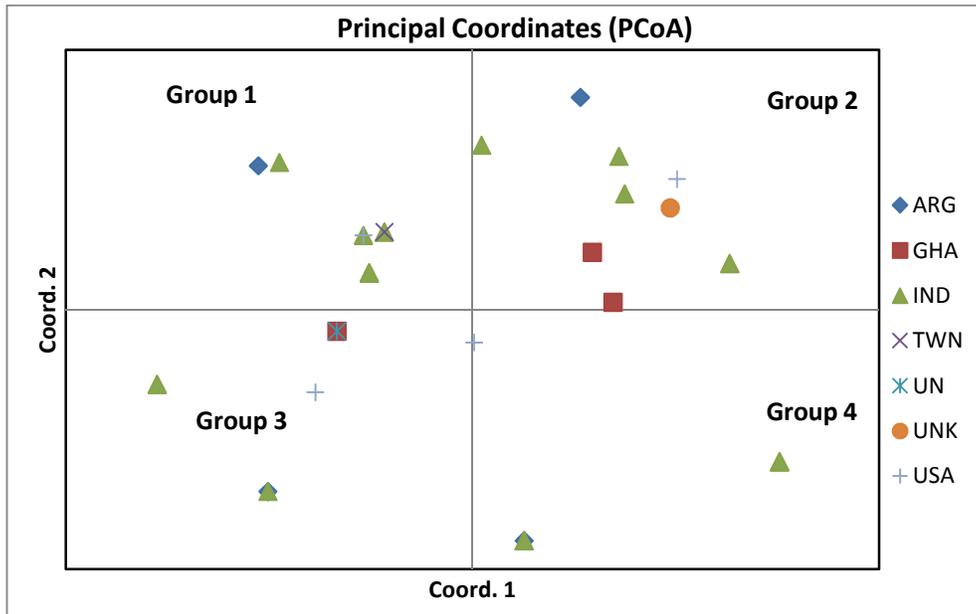


Fig. 2. Principal Coordinates Analysis (PCoA) based on allelic variation using SSR markers representing origin of groundnut germplasm line using GenAlex software

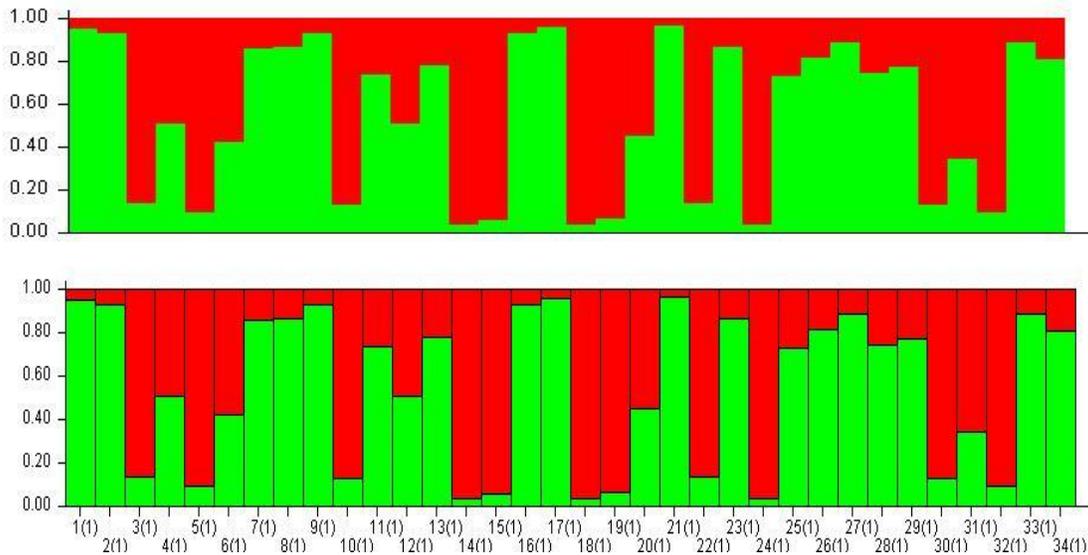


Fig. 3. Single line analysis and multiple line analysis representing two pure and an admixture group of groundnut germplasm lines by Structure 2.3.4 software

3.3 Population Structure Analysis

The population structure of the 34 Groundnut genotypes was estimated using STRUCTURE v2.3.3 software based on SSR markers. The optimum K value was determined by using Structure Harvester, where the highest peak was observed at delta K = 2. The number of sub populations (K) was identified

based on maximum likelihood and delta K (dK) values, with two core and pure groups and an admixture group (Figs. 3 and 4). Using a membership probability threshold of 0.80, 18 genotypes were assigned to group1 (G1), 11 genotypes to G2 and 5 genotypes were assigned to admixture group G3. The relationship between groups derived from STRUCTURE explained that G1 and

G2 comprised of distinct types. This indicated that the population structure was in accordance with clustering of groundnut genotypes formed using UPGMA tree based on SSR data.

Molecular marker analysis on groundnut germplasm using a variety of molecular markers such as microsatellites or simple sequence repeats (SSRs), randomly amplified polymorphic DNAs (RAPDs) and amplified fragment length polymorphisms (AFLPs) in general has shown very low variation in cultivated gene pool because of the evolutionary genetic bottleneck in the form of polyploidy and self-pollination [23, 24,14]. On the other hand, wild diploid *Arachis* species showed relatively higher variation, providing a rich source of genetic variation for genetic and genomic studies. Among different marker systems analysed in the groundnut, like other plant species, SSR markers have been found more informative and useful for genetic analysis and breeding applications [25,26,10,27]. There are several reports on In groundnut gene based markers has been reported for foliar diseases [17,9,28] (Mace et al. 2017). Biotic stresses are one of the major yield limiting factors in groundnut. Foliar fungal diseases of groundnut cause significance yield loss. Chemical control measures are available but

they increase production costs by 10% and are beyond the reach of small and marginal farmers as well as they are not eco-friendly approaches. Development of resistant variety is eco-friendly and cost effective approach. Marker assisted breeding give precise result in less time for resistant variety development. During last decade's molecular breeding approaches has significantly increased genome trait transfer in groundnut improvement for foliar disease with higher yield (Zhou et al. 2016). In peanut, the DNA polymorphism is very low so using genomics assisted breeding can be helpful to enhance peanut productivity. Pandey et al. 2012, have identified a highly informative set of SSR markers using a starting set of 4400 SSR markers and 20 genotypes representing parents of 15 mapping populations. Mapping of quantitative trait loci (QTL) is one of the promising way to identify gene based markers as well as resistant lines of groundnut and has been applied widely for disease resistance [29,30,31, 32,33]. Clevenger et al. [34] used recombinant inbred line population segregating for quantitative field resistance was used to identify quantitative trait loci (QTL) using QTL-seq. This study demonstrates that QTL-seq can be used to rapidly identify QTLs controlling highly quantitative traits in polyploid crops with complex genomes. Pandey et al. [17] suggested

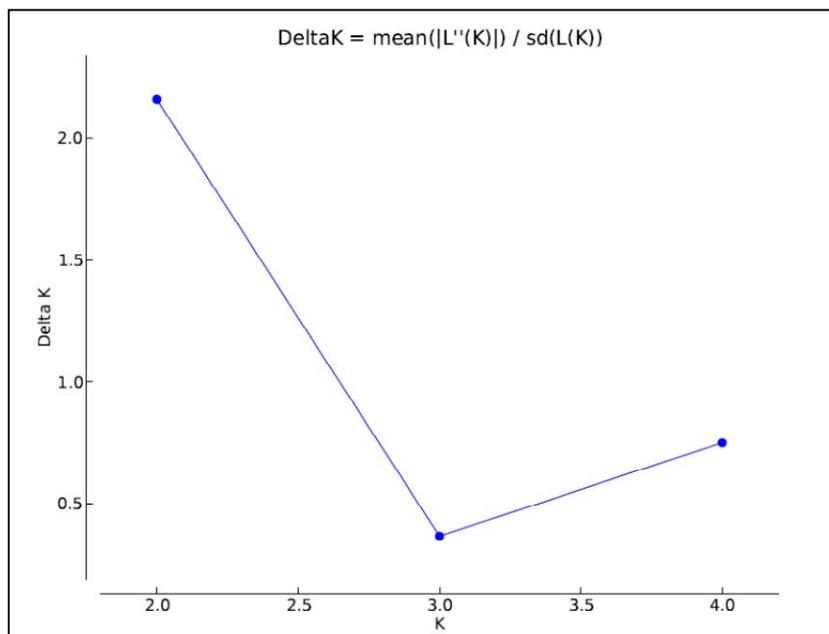


Fig. 4. Delta K value at maximum peak representing number of populations obtained by structure software analysis

usefulness of QTL+seq approach in precise and rapid identification of candidate genomic regions and development of diagnostic markers for breeding applications. They used that approach to locate the genomic region and candidate genes associated with resistance to rust and LLS in groundnut. Recently Chu, et al. [16] developed an RIL population from crossing Florida-07 × GP-NC WS 16 and utilized this population to map QTLs associated with ELS and LLS resistance. For QTL mapping and gene identification, screening of germplasm and identification of donors to make crosses is one of the basic requirement for resistant breeding. Gene based markers are cost effective and easy approach for screening of groundnut germplasm. In our study we used these markers and identified superior germplasm lines which could be used in hybridization programme for mapping and varietal improvement.

4. CONCLUSION

Foliar fungal diseases (LLS and rust) are one of the major limiting factors for groundnut productivity and haulm benefits that provide nutritious fodder for livestock. Development of foliar disease resistant variety is cost effective and environment friendly approach. Resistant variety requires efficient donor for hybridization programme, so germplasm screening is promising approach. Current study identified four germplasms i.e., AH8054, CS21181, CS708, Akola White to be used as donor for groundnut hybridization. The population structure of the 34 Groundnut genotypes was estimated using STRUCTURE v2.3.3 software based on SSR markers. Using a membership probability threshold of 0.80, 18 genotypes were assigned to group1 (G1), 11 genotypes to G2 and 5 genotypes where assigned to admixture group G3. The relationship between groups derived from STRUCTURE explained that G1 and G2 comprised of distinct types. This indicated that the population structure was in accordance with clustering of groundnut genotypes formed using UPGMA tree based on SSR data.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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